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14. ABSTRACT

(Taken From Initiating PI Report) Prostate cancers that recur after so-called androgen ablation therapy ('CR-CaP') are typically more aggressive, more likely to spread to local lymph nodes and bones, and less likely to respond to second-tier treatments, and therefore, contribute to significantly decreased patient survival. We posit that enzymes called Src-family kinases (SFK) are required for the progression to CR-CaP, and thus, targeting these enzymes should prevent CR-CaP formation to suppress their growth. We will use animal models of human and mouse CR-CaP in conjunction with genetic and biochemical experiments to show that SFK are critical to the formation of CR-CaP, and thus, are therapeutically targetable using SFK-specific drugs. Our important pre-clinical studies on the critical role played by SFK in CR-CaP disease will serve as the foundation to establish immediate clinical trials in which CaP patients are treated with drugs such as KX2-391 at the commencement of androgen-deprivation therapy.

15. SUBJECT TERMS

Src, androgen receptor, castration recurrent, human xenografts, SFK isoforms

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Introduction:

From Initiating PI:

We are studying the role of Src-family kinases (SFK) in promoting castration-recurrent prostate cancer (CR-CaP) using genetic and pharmacological approaches, along with several animal models of CR-CaP. Our synergistic collaboration is based upon the expertise of the initiating PI (Gelman) in the molecular signaling of SFK in cancer progression, combined with the expertise of the partnering PIs in the CWR22 and TRAMP CR-CaP mouse models (Mohler and Smith, respectively), and in the role of neuroendocrine cells (NE) in the progression of CR-CaP (Smith).

Body:

The focus of the experimental studies in my laboratory supported by the *Synergistic Research Program* has been to provide expertise in *in vivo* human models of prostate cancer for therapeutic evaluation of inhibitors *of src family kinases* (SFK-inhibitors): particularly, a preclinical model involving primary xenografts of human prostate cancer. Our initial characterization studies were performed in primary cultures of prostate endothelial cells established from clinical specimens as a model for predicting the responses to these inhibitors in primary xenografts of human prostate cancer tissue established from fresh surgical specimens of radical prostatectomy tissue. Importantly, the pre-clinical xenograft model is unique in that it allows analysis of the effects of SFK-inhibitors on all prostate cellular compartments, including an the endogenous human microvasculature in the xenografts that is capable of both angiogenesis and remodeling. Our studies during the tenure of this project (as described in previous Progress Reports) have included: 1) characterization of expression of the individual SFK isoforms, and genes involved in caveolin-1 mediated transmission of growth factor initiated extra-cellular signals, in primary cultures of human prostate endothelial cells isolated from fresh surgical specimens of prostate tissue; 2) characterization of the effects of clinically utilized SFK-inhibitors on angiogenesis/vascular homeostasis in the primary prostate tissue xenograft model; and 3) characterization of the effect of lentivirus-mediated expression of shRNAs specific for individual SFKs on *in vitro* angiogenesis, as evaluated in the HUVEC tube formation assay. The most recent Progress Report detailing much of this data was submitted four months ago (April).

Our hypothesis working hypothesis has been that prostate endothelial cells are key to determining the overall response of prostate cancer, and castration-resistant prostate cancer, to both chemotherapy and androgen deprivation, and that modulation of the function of the endothelial barrier by targeting SRK-mediated processes would dramatically affect cancer growth and progression to castration-resistant disease. Central to this hypothesis is the fact that the prostate endothelial cells are the first cells in the prostate/prostate cancer to encounter systemically administered SFK-inhibitors, and inhibition of SFK-mediated endothelial cell functions could both negatively affect bioavailability of the inhibitors to the prostate cancer tissue and/or contribute to the inhibition of tumor growth of the adjacent prostate cancer cells by perturbation of the endothelial cell transport/barrier functions. Therefore, while the focus of Dr. Gelman's project has been the direct effect of SFK-inhibitors on prostate cancer cells, we hypothesize that the prostate endothelial cells actually represent a/the primary target of these drugs. This hypothesis is supported by data presented in previous Progress Reports that demonstrates marked perturbation by SFK-inhibitors of angiogenesis and vascular homeostasis in the human prostate xenograft model.

Work during the final four months of the project have focused on two issues: 1) characterization of the pattern of gene expression in human prostate endothelial cells freshly isolated by fluorescence activated cell sorting (FACS) from surgical specimens of human prostate and prostate cancer tissue, and comparing the pattern of gene expression in the endothelial cells to prostate cancer specimens; and 2) expansion of studies on the *in*

vitro effects of shRNAs specific for individual SFKs carried in lentiviral expression vectors.

1. Gene expression profile of freshly isolated populations of human prostate endothelial cells harvested from benign prostate tissue and from prostate cancer tissue specimens.

   Fresh surgical specimens of un-involved prostate tissue (benign tissue), and prostate cancer tissue (verified by the Department of Pathology to be >70% cancer), were obtained within two hours of delivery of the excised prostate to Pathology, the tissue enzymatically disaggregated (a 1 hr procedure), and the endothelial cells isolated immediately by two rounds of fluorescence activated cell sorting (FACS). Viable endothelial cells were identified by expression of CD31. RNA was isolated, amplified, and gene expression profiled by array-analysis using Illumina gene expression arrays. This approach was employed, to supplement the data described previously for primary cultures of human endothelial cells, to identify (prevent) artifactual changes in gene expression profile that result from adaptation of the cells to culture. Table 1 presents the gene expression profiling of genes involved in SFK-mediated signaling. Importantly, these pathways are present both in cancer epithelial cells and in prostate endothelial cells, validating the potential of prostate endothelial cells as a significant pharmacologically target. Gene expression profiles are presented for prostate cancer tissue, FACS sorted endothelial cells harvested from benign prostate tissue, and FACS sorted endothelial cells harvested from prostate cancer tissue; the data included in the previous Progress Report for gene expression in primary cultures of human prostate endothelial cells after several weeks in culture is also included for comparison.

   Gene expression profiling of the endothelial cells in primary culture was done using the Agilent array platform, while the expression profiling of the FACS-isolated endothelial cells was performed using an Illumina array platform. Consequently, there are differences in the relative level of "gene expression" (luminence levels) related to the different platforms. However, as anticipated, there were marked differences in the relative level of expression of individual genes between the human prostate endothelial cells in primary cultures and the FACS-sorted fresh endothelial cells, suggestive of gene expression changes associated with adaptation to culture. For example, there appears to be significant up-regulation of expression of Yes-1, AKAP-12, AKT-3, CAV1, KDR, FLT-1, THSB1 and AR in cells adapted to culture compared to the cells freshly isolated from surgical specimens. However, consistent with the data reported in the previous Progress Report, prostate endothelial cells from both benign prostate tissue and prostate cancer tissue express the genes involved in SFK-mediated signaling *in situ* in prostate tissue. In addition, prostate endothelial cells express the majority of these genes at levels comparable to prostate cancer, validating their potential as a target for SFK-inhibitors. Consistent with the data reported in the previous Progress Report that demonstrated that shRNAs for Fyn and Lyn were more effective than shRNAs for src and Yes at blocking endothelial tube formation in the *in vitro* HUVEC assay of angiogenesis, the gene expression profiling of FACS-isolated prostate endothelial cells demonstrated that prostate endothelial cells from both benign tissue and prostate cancer tissue expressed higher levels of Fyn and Lyn than prostate cancer tissue. In contrast, prostate cancer tissue expressed higher levels of src and Yes. Interestingly, Yes actually appeared to increase endothelial tube formation suggesting prostate cancer cells could enhance angiogenic activity by prostate endothelial cells. The most interesting pattern observed in this study is that endothelial cells in prostate cancer tissue express the highest levels of thrombospondin receptors (CD 36 and CD 47), thrombospondin and both hypoxia inducible factors (HIF1α and EPAS1).

In summary, this data validates our hypothesis that SFK-mediated signaling in human prostate endothelial cells represents a realistic, and largely unexplored, target for pharmacologic interdiction in prostate cancer, and that specifically, Fyn and Lyn represent the most logical targets.

TABLE 1. RELATIVE GENE EXPRESSION IN HUMAN PROSTATE ENDOTHELIAL CELLS ISOLATED BY FACS FROM FRESH SURGICAL SPECIMENS OF PROSTATE CANCER TISSUE AND UNINVOLVED PROSTATE TISSUE

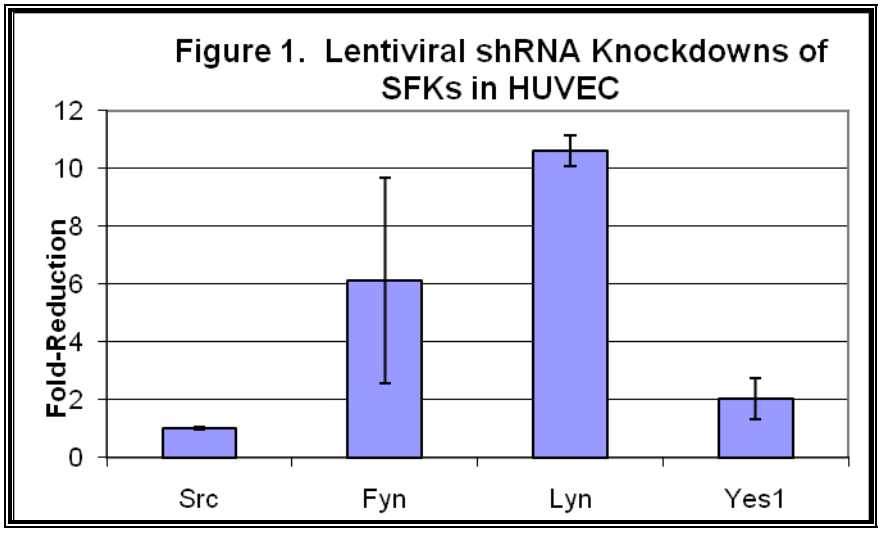
GENE SYMBOL		Prostate Cancer Tissue	Endothelial Cells from Benign Tissue	Endothelial Cells from Cancer Tissue	*Primary Cultures of Prostate Endothelium	DEFINITION
SRC		32.4	5.7	18.7	78.2	v-src sarcoma viral oncogene homolog
FYN		29.0	257.0	185.4	986.8	FYN oncogene related to SRC, FGR, YES
YES1		878.8	209.7	324.6	2056.9	v-yes-1 Yamaguchi sarcoma viral oncogene
LYN		58.9	365.7	511.1	334.1	v-yes-1 Yamaguchi sarcoma viral related oncogene
AKAP12		128.2	77.5	250.9	4170.9	A kinase (PRKA) anchor protein 12 (gravin)
AKT1		941.7	1128.6	879.9	104.4	v-akt murine thymoma viral oncogene homolog 1
AKT2		ND	ND	ND	157.3	v-akt murine thymoma viral oncogene homolog 2
AKT3		16.6	25.5	6.5	2276.9	v-akt murine thymoma viral oncogene homolog 3
NOS3		ND	ND	ND	87.8	nitric oxide synthase 3 (endothelial cell)
CD36		10.1	143.7	224.5	66.0	CD36 molecule (thrombospondin receptor)
CD47		1351.1	524.0	270.2	555.0	CD47 molecule (thrombospondin receptor)
CAV1		1615.9	460.4	827.1	14,200.0	caveolin 1
CAV2		77.0	39.9	165.1		caveolin 2
KDR		4.1	ND	ND	6407.3	vascular endothelial cell growth factor receptor 2
FLT1		ND	ND	24	507.6	vascular endothelial cell growth factor receptor 1
PDGFRA		1212.4	5.3	ND	7.8	platelet-derived growth factor receptor, alpha
PDGFRB		155.8	ND	ND		platelet-derived growth factor receptor, beta
THBS1		871.9	1608.5	1083.6	25453.3	thrombospondin 1
PIK3CA		41.7	39.2	34.3		phosphoinositide-3-kinase, catalytic, alpha
PIK3R1		161.9	327.5	152.1		phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
HSP90		2826.9	3531.9	4436.1	7750.3	heat shock protein 90kDa (cytosolic)
HIF1A		913.2	926.6	1028.5	589.9	hypoxia-inducible factor 1
EPAS1		340.8	175.2	584.4		hypoxia-inducible factor 2
AR		18.8	ND	ND	535.5	androgen receptor

ND = NOT DETECTABLE

*From previous Progress Report

2.<u>Mechanistic evaluation of the action of shRNAs specific for individual isoforms of SFKs on endothelial cell function</u>.

Evaluation of the effect of shRNAs specific for individual members of the SFKs on endothelial tube formation in the MATRIGEL assay suggested clearly that inhibition of Fyn or Lyn activity was significantly more effective at perturbation of *in vitro* angiogenesis than was inhibiting expression of either src or Yes. However, analysis of the knock-down of mRNA levels in HUVEC cells by incubation with the four shRNAs did not produce definitive corroborating data. Figure 1 demonstrates that shRNAs for both Fyn and Lyn knocked down mRNAs levels in HUVEC by 6.2-fold and 10.6-fold, respectively. However, the shRNAs for src and Yes only reduced mRNA levels by 1.0-fold and 2.0-fold, respectively. Therefore, it is not clear whether the lack of inhibition of tube-formation by lentivirus-mediated shRNAs for src and Yes was due to a lack of involvement of these SFKs in tube formation, or, reflected a low level of suppression of mRNA level for these two SFKs. Work during the ensuing extension without additional funds will address this question.



Key Research Accomplishments

- Characterization of the gene expression profile for genes associated with SFK-mediated signaling in freshly isolated human prostate endothelial cells recovered by FACS from surgical specimens;
    - Endothelial cells express equal, or greater levels, of multiple genes in this pathway compared to freshly isolated prostate cancer tissue,
    - Prostate endothelial cells preferentially express Fyn and Lyn, whereas, prostate cancer preferentially expresses src and Yes
- Demonstration that inhibition of Fyn and src significantly suppresses the biological function of human endothelial cells.
- Demonstration that disruption of Fyn- and Lyn-mediated signaling in human prostate endothelial cells represents a high priority candidate for pharmacologic interdiction of prostate cancer.

Reportable Outcomes

None

Conclusion

The project has achieved the major stated goals. During the no cost extension, work will focus on determining which of the SFKs represents the most logical target for interdiction in vivo in primary xenografts with lentivirus expressing the specific shRNA for that SFK-isoform.

References

This work was presented as both a poster and a podium presentation at the CDMRP Innovative Minds in Prostate Cancer Research (IMPaCT Conference) in Orlando, Fl in March 2011.

Appendices

None